

Nitrite Substitutes for Controlling *Clostridium botulinum*

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Nitrite inhibits *Clostridium botulinum* in both culture media and in cured meats such as bacon but it also forms carcinogenic nitrosamines when bacon is fried. A substitute for its antibotulinal activity would therefore be desirable. A search for such a substitute was made, using culture media to study the reaction products of nitrite and ham or bacon for evaluating substitutes. Heating nitrite in culture media causes an increase in its antibotulinal activity; this increased antibotulinal activity against *C. botulinum* was not expressed in the meat environment. An iron-nitrosyl-sulfide compound produced at neutral pH was very active when added to culture media but was not active in meat. A series of 5-nitrothiazoles substituted at the 2-position were very active in culture media and also showed activity in meat. Alkyl esters of *p*-hydroxybenzoic acid were active in culture media but not in meat. The same was true of aliphatic amines and alcohols. Alkenoic and alkynoic acids and their methyl and ethyl esters were active in meat against *C. botulinum*. The esters of fumaric acid were of particular interest. Sorbic acid is a promising substitute for nitrite in meat, particularly under slightly acid conditions. Reduction of water activity (a_w) by dehydration of bacon slices or by increasing the salt content was effective in preventing botulinal toxin formation. Lactic acid production by microorganisms and irradiation are other potential alternatives to nitrite. Although some of the above compounds exhibited antibotulinal activity, none can be used at present as a nitrite substitute with the possible exception of ascorbic acid. Mammalian toxicity studies have not been done on most of these compounds nor have their organoleptic properties been determined.

INTRODUCTION

Clostridium botulinum is a gram-positive, spore-forming, obligatory anaerobic bacillus that produces an extremely potent neurotoxin. Although seven serological types are recognized (A–G), only three (A, B, and E) are associated with the majority of human food-borne botulism cases. Types A and B are of most concern to the food processor since both form extremely heat-resistant spores; type E spores are less heat-resistant and are found only in foods of marine origin. Spores of *C. botulinum*, though occurring in small numbers, are distributed widely in the environment. Any safe food process must, therefore, be based on the assumption that the spores may be present, and under suitable conditions may germinate into toxin-producing vegetative cells.

Nitrite in cured meat products provides an extra measure of protection against the possibility of *C. botulinum* growth under severe abuse conditions (Christiansen et al. 1973, 1974). Nitrite use, however, poses a dilemma. Although inhibiting *C. botulinum* under most conditions, it is also the source of carcinogenic nitrosamines, particularly in bacon on frying (Sebranek and Cassens 1973). A substitute for nitrite for its antibotulinal activity clearly would be useful, at least for bacon. Our laboratory was given the task of finding such an alternative, which is the subject of this report.

The medium used for studies on anticlostridial inhibitors in test tubes was patterned after that of Perigo et al. (1967). Some of the ingredients, however, were found to be unnecessary for either growth of the organism or for studies of heat-induced inhibitors (Huhtanen 1975). A simplified medium was developed that consisted of yeast extract (0.5%), tryptone (0.5%), glucose (0.2%), K_2HPO_4 (0.12%), with either cysteine-HCl, or sodium thioglycollate as a reducing agent. The pH was 6.6. Nitrite was added to the medium either before autoclaving or aseptically after autoclaving ("cold"). A 1.6% (w/v) filter-sterilized solution of $NaNO_2$ was serially diluted (1:1) with sterile water and 0.2 ml from each dilution was added to 5 ml of medium. The final concentrations of $NaNO_2$ were 640, 320, etc., to 1.25 ppm. A "Perigo Index" (the ratio of "cold" vs. autoclaved nitrite inhibition of *C. botulinum*) was used as a measure of the increase in activity caused by heating.

Assay of Compounds in Culture Medium

The medium above was used in assaying various compounds for activity in test tubes against *C. botulinum*. Because many compounds were either insoluble or only slightly soluble in water, they were dissolved in 95% ethyl alcohol, and 0.2 ml was added aseptically to previously autoclaved media, as described above for studies of heat-induced inhibitors. Alcohol alone was not inhibitory unless the concentration exceeded 4%; therefore, the highest concentration of these compounds (1.6% in alcohol) tested was 320 ppm. The minimum inhibitory concentration (MIC) was that showing no growth; however, if a tube showed less than maximum growth, the MIC was calculated as the mean of the tubes showing complete inhibition and partial inhibition.

Studies with Ham

Fresh pork shoulders were purchased locally and were ground twice through a $\frac{3}{16}$ -in plate. Compounds of interest were mixed into aliquots of the meat as described by Huhtanen and Feinberg (1980), using ethanolic solutions if they were water insoluble. No more than 2% final concentration (v/w) of ethyl alcohol was used since alcohol was inhibitory at 4% as in the culture medium. Controls of alcohol, water, and nitrite in water solutions were included in these experiments as in those with culture media.

Studies with Bacon

Two types of studies were done using bacon. One was a screening procedure for antibotulinal compounds; for this purpose a nitrite-free bacon was prepared by a local commercial firm. The target pump was 10 to 15% of green wt with processing to 105% green wt. The pumping brine consisted of (w/w) 84% H_2O , 15% NaCl, 1% sodium tripolyphosphate, and 0.28% sodium erythorbate. The bacon was processed using a normal schedule with a mixture of liquid and natural smoke. The processed bellies were ground twice through a $\frac{3}{16}$ -in plate, were mixed, and were packaged (2 kg per package) in heat-sealed plastic bags. These were kept frozen until needed. Thawing was under running warm tap water. The other type of bacon study was direct pumping of bellies with compounds of interest added to the brine. These studies were done at the laboratory using a hand-stitch pump. The bellies were pumped to 10 to 15% of green wt and were processed in the smokehouse back to original wt. The bacon

was sliced and the slices rearranged to minimize compositional variation (Huhtanen et al. 1981).

Studies with Poultry Frankfurters

Chicken or turkey frankfurter emulsions were obtained from a local processor. These were used as described above for ham, or were commercially processed with additives of interest (Huhtanen and Feinberg 1980).

Inoculation

Clostridium botulinum, type A (NRRL #B-1218, 62A), was used for culture-tube experiments. The culture was grown overnight in the assay medium and was diluted by adding 0.1 ml to 5 ml of the medium. Tubes were inoculated with one drop of the diluted culture.

A mixture of 21 strains of *C. botulinum* (12 type A, 9 type B), prepared as described by Huhtanen and Feinberg (1980), was used for the ham and bacon experiments. For studies using comminuted ham or bacon, spores were mixed into meat aliquots. The meat then was packed into 208 × 107 aluminum-tab cans, which were then sealed utilizing a Rooney canner with 21-in vacuum. The cans were heated to 68 C center temperature for 30 min to activate the spores and to destroy contaminating organisms that could interfere with growth of *C. botulinum*.

Alkenoic and Alkynoic Acids and Esters

These were purchased from either Pfalz and Bauer, Aldrich, or Alfa. For preliminary screening, these were added to the nitrite-free comminuted bacon as ethanolic solutions; three cans were used for each compound. Assessment of activity was based on can swell time (CST) which, in previous experiments with bacon (unpubl. results), had shown that 98.6% of 373 cans which had swelled were toxic. In the same experiments 17.6% of 131 nonswollen cans were toxic. Swollen cans in these screening experiments were not tested for toxin; however, selected nonswollen cans were tested.

Toxin Testing

Pairs of Swiss-Webster mice were injected intraperitoneally with 0.5 ml each of a supernatant obtained by centrifuging a gelatin-phosphate buffer (0.2% gelatin, 0.36% K_2HPO_4 ; 0.15% $Na_2HPO_4 \cdot 7 H_2O$) meat slurry (two parts buffer to one part meat) at 3,000 g for 20 min. Toxic samples were those which produced typical respiratory symptoms of botulism. However, if symptoms were not observed but the mice died, the extracts were boiled for 5 min and were reinjected into other pairs of mice. Extracts were classed as negative for toxin if no symptoms or deaths were observed from the heated extracts.

RESULTS AND DISCUSSION

Effect of Nitrite in Processed Meat on C. botulinum Inhibition

The fate of nitrite added to meat has been studied by a number of workers. Emi-Miwa et al. (1976) reported that although all ^{15}N -labeled nitrite could be recovered in a model system with myoglobin, only 66 to 90% could be accounted for in a meat system. Nitrite recoveries in specific fractions of meat were reported by Gouteffongea

et al. (1977), Woolford and Cassens (1977), Woolford et.al. (1976), and Cassens et al. (1977). The results indicated that 5 to 15% of the added nitrite combined with myoglobin, 5 to 20% was present as free nitrite, 1 to 10% was oxidized to nitrate, 1 to 5% escaped as a gas, 5 to 15% was associated with sulfhydryl groups, 1 to 5% was lipid bound, and 20 to 30% was bound to nonheme proteins.

Residual (free) nitrite, as assayed by the conventional Griess method (Fiddler 1977), decreases rapidly during processing and continues to decrease during storage (Sebranek et al. 1973). In a two-plant study of the effect of storage on nitrite inhibition of *C. botulinum*, though the target level of nitrite was 120 µg/g in the finished bacon, the residual level (4 d after pumping; 1 d after smokehouse processing) was 28 µg/g for bacon from plant D (range of eight bellies was from 16 to 42 µg/g) and 35 µg/g for bacon from plant H (range for eight bellies was 18 to 46 µg/g). After storage at refrigeration (4 C) or freezer (– 23 C) temperatures for 60 d, the residual nitrite levels were, for plant D, 13 and 12 µg/g, respectively; for plant H the levels after storage were 16 and 13 µg/g. These were similar to the reported levels of 46 µg/g before storage and 4 µg/g after 54 d (Christiansen et al. 1974).

These bacon samples were inoculated and canned immediately after processing and again after 60 d at the two storage temperatures. Can swell time with toxin confirmation of selected samples was used as the criterion for growth. The mean swell times (MST's) for cans of bacon from plant D and H, respectively, were: 36 and 41 d immediately after processing; 30 and 36 d after 60 d at 4 C; 15 and 29 d after 60 d at – 23 C. The results did not indicate a close correlation between loss of residual nitrite and increased susceptibility to *C. botulinum* growth.

The meat from different species of animals showed remarkable differences in susceptibility to *C. botulinum* growth. In an experiment comparing chicken- and turkey-frankfurter emulsions with beef (chuck) and pork (shoulders), all with 6.3% NaCl in the aqueous phase, the cans of chicken emulsion without nitrite swelled in 7 d; those of turkey emulsion swelled in 10 d; those of beef in 11 d; and cans of pork swelled in 20 d. With 135 µg/g nitrite, the swell times were 8 d for the chicken emulsion, 12 d for the turkey emulsion, 35 d for the beef, and 71 d for the pork. Mixtures of 50:50 chicken emulsion and pork showed a MST of 11 d, not significantly different from chicken alone. A similar mixture of turkey emulsion and pork showed a MST of 22 d, again not a significant difference from turkey alone. All samples were toxic. Nitrite thus had no appreciable antibotulinal effect in poultry frankfurter emulsions, had slight activity in beef, and had the greatest activity in pork. Fresh sausage also was nearly devoid of a nitrite-inhibitory effect against *C. botulinum*. In one experiment, with no salt addition, 500 µg/g nitrite was needed to show any significant antibotulinal effect.

Heated Nitrite Effect

Perigo et al. (1967) and Perigo and Roberts (1968) showed that nitrite heated in a culture medium had more inhibitory activity against *C. botulinum* than “cold” nitrite added aseptically after autoclaving. In our laboratory (Table 1) the calculated Perigo Index (PI) was 64 for *C. botulinum* 62A when the nitrite was autoclaved (121 C, 20 min) in the medium at pH 7.0. Determinations of PI when components of the basic assay medium were excluded during autoclaving with nitrite showed that without a reducing agent (cysteine) the PI decreased to 4; with cysteine but without either yeast extract or tryptone the PI was 16. The PI was 0.5 when all ingredients were autoclaved separately. The results showed the importance of either yeast extract or tryptone and

TABLE 1. Effect of medium ingredients on heated^a nitrite, Perigo effect

Components Added Separately	Perigo Index ^b
None	64 (320/5)
Cysteine	4 (320/80)
Yeast extract	16 (320/20)
Yeast extract and cysteine	2 (320/160)
Tryptone	16 (160/10)
Tryptone and cysteine	2 (320/160)
Tryptone and yeast extract	2 (80/40)
Tryptone, yeast extract, and cysteine	0.5 (160/320)

^aAutoclaved 121 C, 20 min.^bRatio of unheated/heated nitrite activity against *C. botulinum*.

a reductant for production of Perigo factor. Other experiments (Huhtanen and Wasserman 1975) showed that the addition of excess Fe II or Fe III with "cold" nitrite produced as much inhibition of *C. botulinum* as that from autoclaving nitrite with the medium. Van Roon (1973) implicated iron as a component of an antibotulinal inhibitor in culture media and suggested that black Roussin salt, an iron nitrosylsulfide compound, was similar to, if not identical to, the Perigo factor. A similar compound, (black crystals, ether soluble) possessing a MIC of 0.16 mg/liter, was produced in our laboratory (Huhtanen et al. 1976) by autoclaving (121 C, 10 min) the following solution: 72 mM FeSO₄·7 H₂O, 78 mM Na₃ citrate, 44 mM KH₂PO₄, 172 mM K₂HPO₄, 88 mM sodium thioglycollate, and 145 mM NaNO₂. The pH was 7.0. After cooling, crystals formed which were recovered by decanting the supernatant fluid. Proximate analyses (Huhtanen et al. 1976) indicated that they were crystals of black Roussin salt. The ether solubility of the crystals and their black color also verified their identity. This dried material retained full activity after 2 yr at refrigeration temperature.

Addition of water solutions of the black crystals (iron nitrosylsulfide) to comminuted ham, however, resulted in no increase in antibotulinal activity. Inactivation, presumably by adsorption, was observed immediately upon addition of the compound to the meat; black specks of the compound became visible on the meat. These results indicated that this compound was not the active form of nitrite in meat products. Addition of the Perigo factor ingredients (yeast extract or tryptone and reductant) to meat followed by heating confirmed that the Perigo factor observed from heating nitrite in culture media was not produced in meat.

Excess iron in canned cured pork was found to reverse the inhibitory effect of nitrite (Tompkin et al. 1978), indicating that an iron nitrosylsulfide could be formed which, if inactivated by meat as indicated above, would result in a lessening of nitrite activity against *C. botulinum*.

Nitrosothiols

Nitrosocysteine and nitrosothioglycollic acid were shown by Incze et al. (1974) to possess inhibitory activity against *Salmonella*, *Streptococcus faecium*, and *C. sporogenes*. Mirna and Hoffman (1969) considered such nitrosothiols to be intermediates which could transfer nitroso groups between bacterial cells or spores, or to the meat pigment. Hansen and Levin (1975) found that these nitrosothiols inhibited ¹⁴C uracil incorporation into *Bacillus cereus* RNA. The solution previously described for producing crystals of black Roussin salt, before autoclaving, was a reddish color (probably due to nitrosothiol production) and possessed considerable activity against *C. botulinum*.

in the culture medium (the solution was 1,000 times as inhibitory as an equivalent amount of nitrite alone). The activity slowly disappeared until in 2 wk the activity was the same as the nitrite it originally contained. At this time the color was yellowish. Addition of the reddish solution to the ham system, however, did not inhibit growth or toxin production by *C. botulinum*. Nitrosothiols prepared from cysteine, mercaptoacetic acid, or glutathione and nitrite at pH 1.0 were also devoid of activity against the organism.

Nitrothiazoles

Strong antibotulinal activity was shown by a variety of 2-substituted-5-nitrothiazoles when tested in the medium previously described (Dymicky et al. 1977). The most active of the compounds tested, *n*-lauroylamido-5-nitrothiazole, had a MIC of 0.0025 µg/ml while several others inhibited at 0.01 to 0.4 µg/ml. Some of these (2-amino, 2-succinoylamido, 2-fumaroylamido, and 2-sorboylamido) were tested in the comminuted ham system. At a concentration of 10 µg/g, though prolonging mean swell times (MST) over that of the controls, none were as active as 120 µg/g nitrite. In comminuted nitrite-free bacon (Table 2) at a concentration of 110 µg/g or greater, however, all except 2-fumaroylamido-5-nitrothiazole were more active than nitrite. Some cans containing the fumaroyl or sorboyl derivatives were toxic at this concentration. At 330 and 1,000 µg/g, MST's were greater than 56 d (the duration of the abuse period) and all cans tested were nontoxic except for two of five cans with the sorboyl-5-nitrothiazole at 330 µg/g. These results indicate that the nitrothiazoles possess antibotulinal activity in meat, but at far higher levels than shown in culture medium. The compounds impart a yellowish coloration to the meat; their effect on the taste of treated meat is unknown.

The 2-amido derivative of 5-nitrothiazole has been used in veterinary medicine as an antihistomonad for prophylaxis against blackhead disease in turkeys and chickens. Although once cleared toxicologically for this purpose, the status of it and other 5-nitrothiazoles is now clouded because of recent evidence suggesting that these compounds may be carcinogenic; their use as possible antibotulinal agents in cured meat, therefore, is not being seriously considered at present.

Alkyl Esters of Para-Hydroxybenzoic Acid

The activity of alkyl esters of *p*-hydroxybenzoic acid was demonstrated by Dymicky and Huhtanen (1979). A series of esters was synthesized and tested in culture medium. A plot of ln MIC against chain length indicated that statistically significant quadratic

TABLE 2. Activity of nitrothiazoles against *C. botulinum* spore outgrowth in nitrite-free bacon

Compound	Concentration µg/g					
	111		333		1000	
	MST ^a	Toxic/Tested	MST	Toxic/Tested	MST	Toxic/Tested
2-Amido-5-nitrothiazole	>56	0/5	>56	0/5	>56	0/5
2-Succinoylamido nitrothiazole	>56	0/5	>56	0/5	>56	0/5
2-Fumaroylamido nitrothiazole	18	2/5	>56	0/5	>56	0/5
2-Sorboylamido nitrothiazole	>56	2/5	30(2/5)	2/5	>56	0/5
None	4	5/5				
120 µg/g NaNO ₂	4	5/5				

^aMST = Mean swell time (days), five cans per treatment.

and cubic trends were evident. The most active compounds were those with an alkyl ester chain length of 10 to 13 carbon atoms. A progressive decrease in activity (higher MIC) was noted with chain lengths greater than 11. Several of the more soluble esters were pumped into bellies to give final concentrations of approximately 0.12% in the processed bacon (Table 3). *Para*-hydroxybenzoic acid and its methyl and ethyl esters gave significantly ($P < 0.01$, student t-test) longer MST's than the controls. The MST for the propyl ester-pumped bacon was also significantly different ($P < 0.01$) from its paired control. However, difficulty was experienced in pumping the propyl ester form due to its low solubility in the brine.

Fatty Acid Amides and Alcohols

Huhtanen and Micich (1978) showed that aliphatic amines were inhibitory to *C. botulinum*. Activity was greatest with chain lengths of 14 to 16 carbon atoms, with a suggestion that a reversal of inhibition occurred with an alkyl group of 18 carbon atoms. Inhibition of the organism by aliphatic alcohols was shown by Huhtanen (1980). A very significant quadratic and cubic trend was observed when chain length was plotted against MIC. The most active compounds were those with chain lengths of 13 to 16 carbon atoms (MIC 0.6 µg/ml). Octadecanol showed less activity (MIC 25 µg/ml). Methyl and ethyl alcohols inhibited at a 3% concentration. Some of the most active alcohols were tested at 0.1% levels against *C. botulinum* in the comminuted nitrite-free bacon. No increase in MST over the controls was found for the following alcohols: cetyl, 4-heptyl, stearyl, 2-phenethyl, undecyl, tridecyl, linoleyl, or linolaidyl.

Alkenoic and Alkynoic Acids and Esters

Short-chain, double- or triple-bonded acids and esters were tested at 0.1% levels in the comminuted nitrite-free bacon system for activity against *C. botulinum*. Activity was based on increases in the MST of cans. Strongly inhibitory compounds, i.e., those showing no cans swelling during the 8-wk abuse period, were: maleic acid; methyl-maleic acid; methallyl alcohol; 5-propenoic acid and its methyl and ethyl esters; propiolic acid and its methyl and ethyl esters; monomethyl, monethyl, dimethyl, and

TABLE 3. Effect of benzoate esters on outgrowth of *C. botulinum* spores in bacon

Belly ^a	Addition	Can Swell (Days)			Toxic/ No. Tested
		pH	Mean	Range	
1	None ^b	6.23	18	14-24	10/10
1A	<i>p</i> -Hydroxy benzoic acid ^c	6.35	33	26-48	10/10
2	None	6.39	12	11-14	10/10
2A	<i>p</i> -Hydroxy benzoic acid, methyl ester	6.41	18	15-24	10/10
3	None	6.34	14	13-16	10/10
3A	<i>p</i> -Hydroxy benzoic acid, ethyl ester	6.34	26	21-27	10/10
4	None	6.61	11	11	10/10
4A	<i>p</i> -Hydroxy benzoic acid, propyl ester ^d	6.60	13	11-16	10/10

^aBellies 1 and 1A, 2 and 2A, etc., were matched pairs.

^b1 kg brine contained 100 g H₂O, 150 g NaCl, 50 g sucrose, 5.5 g sodium erythorbate, and 30 g sodium tripolyphosphate.

^cFinal concentrations in processed bacon were 0.12% based on original brine concentrations of 1%, pumping to 110%, and processing to 85% green wt.

^dPropyl ester was very insoluble in brine and clogged screen of the stitch pump. An accurate estimate of its concentration was not possible.

diethyl fumarate. Compounds that were less inhibitory, i.e., approximately equal in activity to 120 µg/g NaNO₂, were: dimethyl glutarate; 4-pentenoic acid; *trans* 2-pentenoic acid; 2,4-hexadien-1-ol; *trans* 2-methyl crotonic acid; vinyl crotonate; *cis* aconitic acid; methyl and ethyl cinnamate; ethylidene acetic acid; and allyl acetic acid. Other compounds which showed no activity, i.e., treated cans swelled as rapidly as the controls, were: 2,4-hexadienoic acid, potassium salt; ethyl-2,4-hexadienoate; *trans* 2-hexenoic acid; *cis* 3-hexenoic acid; ethyl-3-hexenoate; ethyl-2-hexenoate; 2-methyl-2-butenic acid and its ethyl ester; 2-methyl-2-pentenoic acid; 1-penten-3-ol; 4-penten-1-ol; methyl and ethyl crotonate; ethyl, methyl, and dimethyl malonate; diethyl methylmalonate; itaconic acid; glutaconic acid and its diethyl ester; dimethyl succinate; methyl fumaric acid; dibutyl fumarate; 2-ethylhexyl fumarate; dihydroxy fumaric acid; methyl, ethyl, and diethyl maleate; dihydroxy malic acid; diethyl ethylidene malonate; and diethyl allylmalonate.

Maleic acid and propenoic acid and its esters are listed in the *Merck Index*, 9th ed., as having strong irritant properties and probably could not be used in foods. Little is known of the toxicity of most of the other inhibitory compounds although dimethyl fumarate is listed as having a LD₅₀, oral for rats, of 2,240 mg/kg (Registry of Toxic Effects of Chemical Substances, DPHEW 1975). Diethyl fumarate has a LD₅₀ of 1,780 mg/kg according to this source. No conclusions relating structure to activity can be made from these compounds, which were selected on the basis of commercial availability. It appears, however, that the six-carbon compounds were not active at the 0.1% level, while the shorter chain compounds, propenoic and propiolic acid with their methyl and ethyl esters, were inhibitory at 0.04% concentrations.

The four fumarate esters were studied more thoroughly in the nitrite-free bacon system (Table 4). When compared to 120 µg/g NaNO₂, 0.125% levels of the monomethyl and monoethyl esters were more active (no toxic cans in 8 wk compared to five for NaNO₂), while the diethyl ester had activity equal to that of the nitrite.

Sorbic Acid

Sorbic acid (usually employed as the soluble potassium salt) is a GRAS compound although not approved as such for use in meat. Because of its long history of use in many other foods with no untoward health effects, its effect on *C. botulinum* in meat

TABLE 4. Comparison of fumarate esters and nitrite for antibotulinal activity

Addition	Abuse Period (days) ^a							
	7 ^b		14		28		58	
	Cans ^c		Cans		Cans		Cans	
	Swollen	Toxic	Swollen	Toxic	Swollen	Toxic	Swollen	Toxic
None	10	10	ND	ND	ND	ND	ND	ND
120 ppm NaNO ₂	0	1	0	1	0	1	5	6
0.125% MMF ^d	0	0	0	0	0	0	0	0
0.125% DMF	0	0	0	0	0	0	0	0
0.125% MEF	0	0	0	0	0	0	2	0
0.125% DEF	0	0	1	0	1		5	5

^aAbuse temperature 30 C.

^bTen cans per treatment interval; all the rest had five cans.

^cCumulative no. of cans swollen or considered toxic.

^dMMF = monomethylfumarate, DMF = dimethylfumarate, MEF = monoethylfumarate, DEF = diethylfumarate.

ND = Not done.

(sausage) was investigated early in these studies (Tompkin et al. 1974). Other studies had shown sorbic acid was active in poultry frankfurters and emulsions (Sofos et al. 1979; Huhtanen and Feinberg 1980); bacon (Ivey et al. 1978), and comminuted pork (Ivey and Robach 1978). The undissociated acid is more active than the dissociated form; therefore, lowering of pH will greatly enhance its efficacy. In bacon this can be done by acidification of the pumping brine or, as shown by Huhtanen et al. (1981), a very finely pulverized, though insoluble, form of sorbic acid can be pumped under regular commercial conditions. In the latter study, when the concentration of sorbic acid in the processed bacon exceeded 0.13%, there were no swollen or toxic cans during the 6-mo abuse period.

Several experiments were conducted comparing a number of acids for their potentiating effect on sorbic acid or potassium-sorbate inhibition of *C. botulinum* (Huhtanen et al. 1983). In one experiment using comminuted nitrite-free bacon with or without 0.1% sorbic acid (a level chosen as one calculated to be without antibotulinal activity from previous work), the enhancement of activity by hydrochloric, phosphoric, acetic, citric, lactic, and succinic acids was studied. In each case the quantity of acid added was such that the pH values of all samples were nearly equal (approx. 5.75). In this experiment, the acids alone had no effect on the number of cans that became toxic (cans incubated at 30 C were tested at 10, 17, and 31 d). Sorbic acid alone, as expected, showed no activity. With the acids plus sorbic acid, there were fewer toxic cans at 10 d. At 17 and 31 d all cans were toxic. Thus, in bacon, the undissociated acid per se did not increase effectively antibotulinal protection on sorbic-acid activity against *C. botulinum*.

The effect of sorbic acid in comminuted ham was different from that in bacon. Again the acids, at pH's of approximately 5.8, had no effect on the number of toxic cans. Sorbic acid at 0.1% showed no toxic cans in 1 wk; in 2 wk, three of five cans were toxic. Citric, lactic, and succinic acids plus sorbic acid showed, respectively, one, two, and two toxic cans in 2 wk, while HCl, H₃PO₄, and acetic acid showed none. At 4 wk, only cans of ham treated with acetic acid plus sorbic acid remained free of toxin.

The effect of two levels of phosphoric acid (0.04 and 0.08%) on the effectiveness of sorbic acid or potassium sorbate in comminuted ham was studied in another experiment. Phosphoric acid alone had no effect on the number of toxic cans, nor did sorbate or sorbic acid at 0.26 and 0.20%, respectively. Sorbate at 0.52% or sorbic acid at 0.40%, however, completely inhibited toxin production for 180 d, the duration of the abuse period. At the lowest level of sorbate or sorbic acid (0.10 or 0.08%, respectively) phosphoric acid had no effect, but at 0.26% sorbate, 0.08% phosphoric acid showed fewer toxic cans at 180 d (2/5 vs. 5/5 for those without acid). With 0.20% sorbic acid, there were no toxic cans with either 0.04 or 0.08% phosphoric acid. The pH's at these concentrations were 5.46 and 5.42, respectively. These experiments indicated that protection against *C. botulinum* requires at least 0.52% potassium sorbate or 0.40% sorbic acid; these levels can be halved if a modest amount of phosphoric acid also is added.

A study of the commercial feasibility of sorbate use in bacon was conducted by the USDA (1979) using four commercial bacon processors. Bacon was prepared with 120 µg/g nitrite, 40 µg/g nitrite plus 0.26% potassium sorbate, or with neither addition. The combination of low-level nitrite and sorbate provided antibotulinal protection approximately equivalent to that of 120 µg/g nitrite.

The control of *C. botulinum* in meat products could be effected by the addition of salt alone, but the concentration required would render most such products unpalatable. The consequences of reducing the salt concentration in the case of *C. botulinum* type A in a laboratory medium are illustrated by the experiments of Roberts and Ingram (1973). The *C. botulinum* grew in 6% sodium chloride but not when 50 µg/g nitrite also was present. Reducing the salt to 4% allowed growth even in the presence of 50 µg/g nitrite, but not with 100 µg/g. Further reduction of salt to 3% allowed growth even in the presence of 100 µg/g nitrite. The importance of salt for inhibition of *C. botulinum* in commercially prepared bacon was shown in an experiment in our laboratory. Packages of bacon representing eight different bellies were obtained from the production lines of three local processors. The slices were rearranged as previously described to minimize variation and were ground and mixed. Moisture-phase salt concentration (MSC) and MST's were determined. Plant D used a brine of 11.4% salt; plant M used 12.5%; while plant H used 17.7%. Nitrite was used at 0.12%. The average, target pump rates were about 12% in each plant, with processing to slightly over green wt. The MSC for plants D, M, and H were, respectively: 5.08% (range 4.33 to 6.28); 5.44% (range 3.25 to 10.05); and 6.13% (range 4.88 to 7.80). The MST's of the inoculated cans were, respectively, 31, 41, and 45 d. Analysis of variance and the Duncan Test showed that both MSC and MST of plant D were significantly different from the other plants. Coefficients of variation (CV) of MSC were (for plants D, M, and H, respectively) 12.7, 37.7, and 18.2. These large CV's illustrate the difficulty in obtaining uniform pumping, within or between plants.

The importance of salt for *C. botulinum* inhibition also was shown in comminuted ham. Four levels of salt, calculated on the water phase, were 4.5%, 5.0%, 5.5%, and 6.0%. The MST's and ranges, in parentheses, were, respectively: 9 (6–11), 13 (12–13), 18 (12–24), and 47 (14–63). All cans were toxic. A comparison of two levels of NaCl, 5.30 and 6.33% in the moisture phase, with replacement of 1/5 or 1/3 of the NaCl by an equivalent quantity of KCl, showed no differences in MST or number of toxic cans (all swollen cans were toxic) attributable to KCl, but the lower MSC showed a MST of 15 d while the higher MSC showed a MST of 46 d. These cans were also toxic.

Other Inhibitors of C. botulinum

Four 3-0-alkanoyl glyceric acids (C_{10} , C_{12} , C_{14} , and C_{16}) were tested in nitrite-free comminuted bacon (five cans each) at concentrations of 0.20% (Table 5). None showed any appreciable increase in MST over the controls. All cans tested were toxic.

Several antioxidants (ascorbic acid, isoascorbic acid, butylated hydroxy anisole, butylated hydroxytoluene, propyl gallate, and α -tocopherol) were tested also at concentrations of 550 µg/g in bacon with and without 25 µg/g NaNO_2 . None showed any increase in MST over the controls; all cans were toxic.

Nisin is a polypeptide produced by certain strains of *Streptococcus lactis* which has been shown effective in preventing spore outgrowth (Lipinska 1977). Scott and Taylor (1981) showed that nisin inhibited types A and B *C. botulinum*-spore outgrowth in a liquid medium but could not demonstrate significant activity in a cooked-meat medium. Several experiments in the comminuted nitrite-free bacon failed to show any activity against the organism at levels as high as 1,500 µg/g.

Alcoholic extracts of spices were tested in the assay media for activity against *C. botulinum* (Huhtanen 1980). Extracts of mace and achiote were the most inhibitory; bay leaf, white and black pepper, and nutmeg extracts were also active. Whole ground spices at concentrations of 0.1% or equivalent alcoholic extracts were tested in comminuted ham but none showed any increase in MST over the controls.

Sodium hypophosphite is a relatively nontoxic compound which has been studied by Pierson et al. (1981) as a possible substitute for nitrite in bacon. Prolongation of swell times and time for toxin production were reported at sodium hypophosphite levels of 0.1% or higher. It was much more effective when used in combination with 40 $\mu\text{g/g}$ NaNO_2 .

Irradiation is a promising nitrite substitute (Rowley and Brynjolfsson 1980). Anellis et al. (1972, 1977) demonstrated the antibotulinal efficacy of corned beef or ham prepared with low levels of nitrite (25 $\mu\text{g/g}$) and irradiated at 4 Mrad. Rowley et al. (1983) showed that irradiation of bacon containing 40 $\mu\text{g/g}$ nitrite at 1.0 or 1.5 Mrad significantly delayed onset of toxic spoilage when compared to nonirradiated control bacon with 120 $\mu\text{g/g}$ (target level) nitrite.

Acidification can be used to prevent *C. botulinum* growth. No strains have been observed to grow under normal conditions at a pH less than 4.77 (Hauschild et al. 1975). Our laboratory conducted a study using glucono- δ -lactone (GDL) as an acidulant in comminuted ham. Before adding GDL the pH was 5.9; the MST of this control ham was 4 d. The GDL was added in 0.2% increments from 0.4 to 1.8% and the pH's were determined on noninoculated cans. All cans swelled and were toxic at 1.0% GDL (pH 5.2); at 1.2% GDL (pH 5.1), $\frac{2}{3}$ cans swelled, both were toxic; at 1.4% GDL (pH 5.1) $\frac{1}{3}$ cans swelled, but it was not toxic. No swollen or toxic cans were found at 1.6 or 1.8% GDL (pH's were 5.0 and 4.9, respectively).

Reduction of water activity (a_w) can be used to prevent growth of *C. botulinum*. This can be done by adding extra salt to the curing brine, but the same result can be achieved by removing moisture from the finished product. In the case of bacon, this results in a product that has no more salt per slice than the original, but removal of enough water will prevent growth of *C. botulinum* without the necessity of adding high levels of nitrite. Enough nitrite, about 30 $\mu\text{g/g}$, can be added to give the characteristic cured meat color. In laboratory experiments, bacon slices were dried at 70 C in a forced-draft oven. If drying was slow enough, evaporative cooling prevented

TABLE 5. Effect of 3-0-alkanoyl glyceric acids on *C. botulinum* spore outgrowth in ham

Compound	Can Swell Times (days)	
	Mean	Range
None	10	9-12
120 $\mu\text{g/g}$ NaNO_2	49	22-65
0.2% 3-0-decanoyl glyceric acid	14	13-16
0.2% 3-0-dodecanoyl glyceric acid	11	9-12
0.2% 3-0-tetradecanoyl glyceric acid	12	12
0.2% 3-0-hexadecanoyl glyceric acid	12	10-12

Five cans per treatment; all cans tested (two from each treatment) were toxic.

rendering of the fat. The dried slices very closely resembled the original and, after frying, were not organoleptically different from the starting material. In a series of seven slice-drying experiments, the a_w was monitored with lithium chloride sensors (AMINCO). Drying time varied depending on the moisture content of the samples, but no growth or toxin production was observed at any a_w below 0.92. At an a_w of 0.94, cans swelled and were toxic, although swelling was delayed up to 134 d. In another experiment, comminuted, nitrite-free bacon was dried by lyophilization and water added back to give a_w 's of 0.90 to 0.96. No growth (swelled cans) or toxin production was found below an a_w of 0.94.

Acid production by inherent or added lactic-acid bacteria may be effective in prevention of *C. botulinum* growth. Tanaka et al. (1980) reported that bacon prepared with adequate sucrose (0.9% target level) and inoculated with *Lactobacillus plantarum* effectively inhibited growth of *C. botulinum*. The indigenous lactic-acid bacteria cannot be relied on, however, to produce sufficient acid. Results with noninoculated bacon samples indicated that, even with adequate sucrose, sufficient acid was not always produced to provide adequate antibotulinal protection.

SUMMARY

Many of the compounds used in our studies are not GRAS, and their use is restricted to experimental studies. Some appear to hold enough promise as antibotulinal nitrite substitutes to warrant at least limited, acute toxicological testing, i.e., the fumarate esters, but the other alkenoic and alkynoic acids and esters appear to be too irritative, corrosive, and possibly toxic to warrant the expense of comprehensive toxicological testing. Sorbic acid probably could be used commercially since it is effective and has a long history of problem-free use in the food industry. The simplest antibotulinal agent would be an increase in the salt content of the brine; however, because of the great variation in pumping rates, this would probably result in many bacon samples being too salty. The activity of sodium hypophosphite needs more study. The use of lactic-acid bacteria also requires more study since their use may cause untoward organoleptic properties.

NOTE: Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

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